2073

THE ACTION OF HEAT AND MOISTURE ON LEATHER

V. CHEMICAL CHANGES IN COLLAGEN AND TANNED COLLAGEN*

J. H. Bowes and A. S. Raistrick

British Leather Manufacturers' Research Association

Milton Park, Egham, Surrey, England

ABSTRACT

Collagen from the center split of a limed oxhide was tanned with formaldehyde, two levels of chrome tan, and three vegetable tans, myrobalans, mimosa, and sulfited quebracho. The collagen and leathers were stored over water for 6 weeks at 60°C. and 16 weeks at 40°C. The specimens were examined for buckle tear strength, solubility of hide substance in acetic acid, changes in shrinkage temperature, pH of water extract, amino acid composition, and N-terminal amino groups. The applicability of these methods to leathers is considered.

Breakdown of the protein apparently occurs both by hydrolysis and by some other, presumably oxidative, mechanism. The amount of hydrolytic breakdown is dependent on the pH of the leather, as would be expected, but the resistance to deterioration under warm moist conditions also depends on the effectiveness of the tanning material in preventing loss of molecular organization following the breakdown.

INTRODUCTION

Previous papers in this series (1–4) have reported the effect of storage at high humidities and temperatures of 40°C. and 60°C. on some of the physical and chemical properties of a wide range of leathers. Large increases in solubility of hide substance in $5M$ acetic acid were found in some instances (2–4), but other leathers which were obviously seriously damaged still had low soluble-nitrogen figures. Therefore it was clear that this was not satisfactory as a single method for determining breakdown.

The present paper is primarily concerned with the study of the breakdown of the hide protein during storage and the effect on this of various tanning

*This work forms part of a program of research sponsored by the U.S. Dept. of Agriculture under the authority of Public Law 480.

agents. Hydrolytic attack on peptide bonds is a probable cause of degradation of skin protein. Such breakdown during liming (5) and the production of gelatin (6) has been followed using the fluorodinitrobenzene method to determine the α-amino groups released. The possibility of using this method was very attractive, especially if it could be applied to leathers.

Cassel (7) found that storage of collagen at 100°C. to 170°C. for 3 days resulted in a reduction in the amounts of several amino acids, suggesting that breakdown occurred by some mechanism other than hydrolytic scission. It was thought that long periods of storage at lower temperatures might also produce changes in the amino-acid composition of collagen or leather, so this aspect was also followed.

As this investigation was concerned with chemical rather than physical changes, the material used was prepared from the center split of an oxhide so that complications due to the various structures in the grain layer would be avoided. For the sake of simplicity this material is referred to throughout as collagen.

EXPERIMENTAL

Materials.

Collagen.—The butt of a freshly flayed oxhide was green-fleshed, cut into pieces about one foot square, and washed with water. The pieces were drummed for 2 hours with each of four changes of a 300% float of 5% salt, washed in water to remove salt, and limed for 1 week in a suspension of freshly slaked lime with 0.1% sodium sulfide added. The hair was scraped off, and after deliming to pH 4 with acetic acid, the hide pieces were well washed in tap water, followed by two changes of distilled water. They were then acetone-dehydrated, and finally the flesh and grain layers were split off.

For tanning, this material, approximately 4 mm. thick, was cut into pieces about 3″ x 1½″. For each tannage 240 g. was soaked back in distilled water for 24 hours, giving a drained wet weight of 560 g.

Formaldehyde tannage.—The collagen was shaken with 1 l. of 5% sodium sulfate for 1 hour. Fifty ml. of 40% formalin was added (giving a final concentration of about 2% formaldehyde), and shaking was continued for 2 hours. The solution was slowly adjusted to pH 8.5 with 10% sodium hydroxide solution, and shaking was continued for a further 24 hours. The pieces were piled for 24 hours, washed 2 hours in 1% ammonium sulfate solution, and then washed in water until no sulfate was detected in the washings, using barium chloride solution. The pieces were finally acetone-dehydrated.

Chrome Tannage.—Chrome-tanning was carried out at two levels, the chrome tan offered being either 1% or 4% Cr_2O_3 on drained soaked weight.

Two batches of collagen were pickled to pH 3 with sulfuric acid in a 1 l. of 5% sodium sulfate for 24 hours. To one batch was added 70 ml. and to the other 280 ml. of a 33%-basic SO_2 - reduced chrome liquor containing 8% Cr_2O_3. After shaking for 1 hour they were left overnight, and the following day they were very slowly basified to pH 4 with sodium bicarbonate. The leathers were left in the liquors for 3 days, with occasional shaking, and then piled one day. They were washed in several changes of tap water, followed by distilled water, and finally dehydrated in acetone.

Vegetable Tannage.—Three vegetable tanning materials were used, myrobalans, mimosa, and sulfited quebracho. Liquors 50°Bk, with natural salt content, brought to pH 3.5 with sulfuric acid, were prepared. The pieces of collagen were shaken for 8 hours with 1200 ml. of the appropriate liquor and were then suspended for 10 days in a fresh batch of liquor, the liquors being mended to 50°Bk and corrected to pH 3.5 with sulfuric acid daily. The pieces of leather were given a quick rinse in water and dehydrated in acetone. In all cases the acetone-dehydrating procedure appeared to remove considerable amounts of tan from the leathers.

Methods.

Storage of samples for deterioration.—Samples of the collagen and leathers were stored suspended over water in closed glass tanks (i.e., roughly 100% r.h.) in incubators at either 40°C. or 60°C. The tanks kept at 40°C. also contained a small amount of toluene to discourage mould and bacterial growth.

Analysis of leathers.—The samples for analysis were cut into about 2-mm. squares or ground to pass a 20-mesh sieve, whichever was more convenient.

Moisture, grease, water-soluble matter, ash, hide substance, and chrome content were determined by the official SLTC methods. Buckle tear loads were determined by the official SLTC method except that the samples were about 3″ x 1½″.

Formaldehyde in leather was determined by the method described in *Progress in Leather Science* (8).

Shrinkage temperatures were determined in water, except for the chrome leathers which were determined in 75% v/v glycerol. All the samples were soaked back in distilled water first.

pH of water extract was determined by the standard method of ALCA.

Total soluble nitrogen and volatile nitrogen extracted by $5M$ acetic acid and $0.1M$ acetic acid were determined as described in Part II of this series (2).

N-Terminal residues.—N-Terminal residues were determined by a modification of the fluorodinitrobenzene (FDNB) method of Sanger (9). The material on which the determination was to be made was ground to pass a 20-mesh sieve and suspended in saturated sodium bicarbonate, 20 ml. per gram of sample. FDNB, 0.5 ml. per gram of sample, was added, and the mixture was shaken gently for 48 hours. It was then carefully acidified with 20% HCl, excess FDNB was removed by extraction with ether, and the solid was filtered off and washed with water, alcohol, and finally, ether.

It was found that the presence of alcohol in the reaction mixture as specified in the original method resulted in wide variations in the amounts of dinitrophenyl–amino acids (DNP–amino acids) found, and the product appeared to be partially gelatinized, suggesting that some breakdown of the protein had occurred. The method adopted gave rather lower amounts of DNP–amino acids, but the results were more reproducible, and the product did not show any gelatinization.

Between 0.5 g. and 4 g. of the DNP-collagen or leather, depending on the amounts of DNP–amino acids expected, was hydrolyzed, and the DNP-amino acids were separated from the hydrolyzate as described by Bowes and Moss (5).

The DNP–amino acids and dinitrophenol were separated by the method of Courts (6), slightly modified. DNP-glycine, DNP–glutamic acid, DNP-serine, DNP-threonine, and DNP–aspartic acid were retained on a 4.5 g. column of Celite 545 buffered at pH 4, using chloroform as eluant, and were washed off in turn, using increasing amounts of ether added to the chloroform. The other DNP–amino acids and dinitrophenol were separated on a second column buffered at pH 7, using chloroform, ether, and finally 2% acetic acid in ether as eluants. Solvents used were analytical reagent grade, well washed with water.

The solvents were evaporated off under reduced pressure; the DNP–amino acids were then dissolved in 1% sodium bicarbonate in 25% ethanol, and the absorption of the solutions at 360 mμ was measured. The amounts present were determined from standard curves prepared using samples of DNP–amino acids, prepared from the free amino acids using the methods of Sanger (9), Porter and Sanger (10), and Rao and Sober (11). These DNP–amino acids were also used for determining the R_f values on the columns.

Correction factors for the loss of a few of the DNP–amino acids during hydrolysis were obtained by adding a known amount of the pure DNP–amino acid to a sample of collagen, and hydrolyzing and separating as above. The values obtained agreed with those of Bowes and Moss (5), and their factors were taken for the other DNP–amino acids.

For the determination of ϵ-DNP-lysine 10 mg. of the material was taken for hydrolysis with 1 ml. of conc. HCl, as above, and the hydrolyzate was evaporated to dryness on the steam bath under vacuum. Two ml. water was added to the residue, which was again evaporated to dryness to remove the HCl. The residue was then extracted with a mixture of methyl-ethyl-ketone/ether (2:1), and the organic solution transferred to a 1.5 g., 1-cm.

diameter column of Celite 545 buffered with 0.75 ml. of buffer, pH 5.3, (68 ml. 0.2 MNa_2HPO_4 + 32 ml. 0.2M citric acid). The eluant was methyl-ethyl-ketone/ether (2:1).

It was found that on a column buffered at pH 7.1, described by Courts (6), the ϵ-DNP-lysine decomposed spontaneously, the R_f of the band changing from 0.2 to 1.1 during movement down the column. At pH 5.3, however, this was not observed and the R_f of ϵ-DNP-lysine was found to be 0.4.

The effluent was collected and evaporated down at the pump and taken up in 1% HCl 25% ethanol, and the ϵ-DNP-lysine was determined by measuring the absorption at 360 mμ. A second band running closely behind the ϵ-DNP-lysine was shown to have the same R_f as a known sample of ϵ-DNP-hydroxylysine. There was not sufficient of this amino acid available for the preparation of a standard curve, so it was not determined. The amounts present, however, appeared to vary in the same way as the ϵ-DNP-lysine.

The results were calculated as millimoles per hundred grams of protein in all cases.

Amino acid analysis.—Samples of collagen and some of the leathers were analyzed for amino acid content by the method of Moore, Spackman, and Stein (12), as modified by Hannig (13), using a Bender and Hobein automatic amino acid analyzer. The determination of the basic amino acids in some of the DNP-materials was conveniently carried out on the IRC-50 column as used by Hannig, the DNP–amino acids and artifacts not being retained on the column.

The samples were hydrolyzed with 6N HCl in sealed tubes for 18 hours at 105°C., and the excess HCl was removed by evaporation on a water bath. Hydrolysis of all the samples appeared to be complete, as no unidentified peaks due to peptides were observed.

RESULTS

Appearance of leathers, and analysis by standard methods.— The analyses of the collagen and leathers before storage are given in Table I.

After 17 days at 60°C. the myrobalans-tanned leather had lost most of its fiber structure and was dripping into the bottom of the tank as a dark viscous fluid. It was therefore removed from storage. After 24 days at 60°C. the mimosa-tanned leather was in a similar condition and was also taken out of storage. The quebracho-tanned leather did not reach this condition until it had been stored for six weeks, when all the samples at 60°C. were removed. At this time the only other material showing obvious breakdown of fiber structure was one of the pieces of chrome leather of low chromic oxide content which had a glassy layer in the middle about 0.5 mm. thick. All the vege-

TABLE I

ANALYSIS OF COLLAGEN AND LEATHERS

(g/100 g. air-dry weight)

	Collagen	Formaldehyde	Low Chrome	High Chorme	Myrobalans	Mimosa	S. Quebracho
Moisture	14.4	14.7	13.7	13.6	11.9	13.5	11.8
Grease	0.2	0.1	0.1	0.3	0.1	0.1	0.1
Water sols.	0.01	0.01	1.3	1.4	4.9	2.7	2.2
Ash (total)	0.1	0.1	2.0	5.5	0.2	0.2	0.2
Hide substance	85.5	84.6	81.9	71.3	64.5	62.0	58.3
Cr_2O_3	—	—	1.3	4.6	—	—	—
Formaldehyde	—	0.65	—	—	—	—	—
Fixed tan (by difference)	—	—	—	—	18.6	21.6	27.6
Insoluble ash	—	—	1.7	5.1	0.1	0.1	trace
Degree of tannage	—	—	—	—	29	35	47

table- and chrome-tanned leathers were very cracky and weak. Untanned collagen, however, still retained over half its buckle tear load and showed only slight hardening and yellowing. The formaldehyde-tanned leather still retained 95% of its original buckle tear load, although only one quarter of the original formaldehyde was recovered by analysis.

After 16 weeks at 40°C. over water the vegetable-tanned leathers were darker in color than originally, but not so dark as those stored at 60°C. They had all lost strength, the myrobalans-tanned retaining about 40%, the mimosa 70%, and the quebracho 60% of their original buckle tear loads. Both the high- and low-chrome-content leathers showed little change in appearance and retained 70% of their strength. The formaldehyde-tanned leather was unchanged in appearance and had actually increased slightly in strength. The collagen had lost about half its strength; the pieces had curled up considerably and had become hard and rather brown in color, but if the pieces were flexed, their appearance became quite good.

The results of shrinkage temperature and pH of water-extract measurements are given in Table II. There was a general tendency for the shrinkage temperatures to go down, but in a few cases there was an increase. In general the pH of water extract increased, but the increases varied greatly.

Soluble nitrogen.—The results of the determination of total nitrogen and volatile nitrogen extracted by 0.1*M* and 5*M* acetic acid are given in Table III.

As was expected from previous results described in Parts II (2) and III (3), the nitrogen dissolved from the leathers by 5*M* acetic acid did not become really high until the fiber structure was lost. Again, as in the earlier results,

TABLE II

SHRINKAGE TEMPERATURES AND pH OF WATER EXTRACT

Tannage	Ts(°C.) Original	Ts(°C.) Stored at 40° C.	Ts(°C.) Stored at 60° C.	pH Original	pH Stored at 40° C.	pH Stored at 60° C.
Collagen	60	56	58	4.6	5.8	4.8
Formaldehyde	78	77	73	4.6	6.4	6.4
Low chrome	83	75	81	3.1	3.4	3.1
High chrome	106	99	110	3.0	3.1	3.3
Myrobalans	70	62	—	3.1	6.1	3.6
Mimosa	82	85	—	3.5	3.4	3.6
S. quebracho	77	80	—	3.2	4.3	4.6

TABLE III

NITROGEN SOLUBLE IN 0.1M ACETIC ACID OR 5M ACETIC ACID

(*% of total nitrogen*)

Tannage	Concentration of Acetic Acid Used as Solvent	Original		Stored at 40° C. for 16 Weeks		Stored at 60° C. for 6 Weeks*	
		Total Soluble	Volatile	Total Soluble	Volatile	Total Soluble	Volatile
Collagen	0.1M	0.17	0.01	15.4	1.5	8.7	0.08
	5M	0.73	0.01	20.3	1.6	20.0	0.17
Formaldehyde	0.1M	0.12	0.06	0.22	0.04	0.18	0.04
	5M	0.16	0.07	0.27	0.06	0.37	0.07
Low chrome	0.1M	0.09	0.05	1.1	1.0	3.5	0.62
	5M	0.21	0.05	1.3	1.1	3.9	0.68
High chrome	0.1M	0.10	0.07	0.69	0.54	1.1	0.84
	5M	0.21	0.08	0.80	0.65	2.1	1.0
Myrobalans*	0.1M	0.19	0.04	7.3	7.1	5.6	0.37
	5M	1.9	0.05	9.3	8.1	23.2	0.39
Mimosa*	0.1M	0.08	0.03	0.82	0.50	3.8	0.64
	5M	0.69	0.02	0.92	0.60	22.1	0.82
S. quebracho	0.1M	0.23	0.05	1.1	0.39	1.6	0.50
	5M	1.1	0.06	1.6	0.85	18.2	0.55

*Myrobalans-tanned leather disintegrated after 2½ weeks at 60° C. Mimosa-tanned leather disintegrated after 3½ weeks at 60° C.

this only occurred with the leathers containing vegetable tannins. Although the fiber structure of the collagen was not lost, the fibers appeared stuck together, and the soluble nitrogen was high after storage at both 40°C. and 60°C. If the 5M acetic acid extract of the collagen stored at 60°C. was diluted with water, neutralized, or dialyzed against water, a gelatinous precipitate was formed.

The high volatile nitrogen of the leather tanned with myrobalans and stored at 40°C. falls rather out of place and probably accounts for the unusually high pH of water extract of this leather. Two possible explanations are either some specific action of this vegetable tan or damage by bacteria. The volatile nitrogen of the leather stored at 60°C. was not high, so the first explanation seems unlikely. On the other hand, with the other leathers, and even with untanned collagen, the toluene was apparently successful in preventing bacterial damage, so that also seems unlikely as the cause of the high volatile nitrogen.

The outstanding behavior of the formaldehyde-tanned leather is particularly interesting. Although the original strength was the lowest of the leathers, no loss in strength occurred, and the soluble nitrogen was virtually unchanged by the storage, even though the formaldehyde content was reduced from 0.65% to 0.13%. The increase in pH of water extract is difficult to account for in view of the small changes in soluble nitrogen, but it may be related to loss of formaldehyde and consequent release of amino groups.

N-Terminal residues.—If hydrolytic breakdown of the collagen in leather occurs during storage, α-amino groups will be released, and these should be detectable with FDNB, provided that the tans do not interfere with the reactions involved. They may do this either by preventing the reaction of FDNB with the α-amino groups because of steric factors or by destroying the DNP-amino acids during the hydrolysis of the treated leathers.

Recovery experiments were carried out by hydrolyzing DNP-glycine and ϵ-DNP-lysine with samples of chrome-tanned and vegetable-tanned leathers. No differences were detected from similar recovery experiments using untanned collagen, so it was considered that the chrome and vegetable tans did not affect the recovery of the DNP–amino acids.

The effect of the tan on the accessibility of liberated terminal amino groups was more difficult to assess. All three types of tanning material, vegetable, chromium, and formaldehyde, reduced the number of N-terminal residues found (Table IV), due, at least with the formaldehyde, to reaction with α-amino groups. With the formaldehyde- and chrome-tanned leathers, particularly the latter, there were increases in terminal residues during storage. Repeated extraction of a chrome leather with sodium citrate until the chromium content was reduced below 0.2% had no significant effect on the number of N-terminal residues found. The method, therefore, seems applicable to both chrome and aldehyde leathers. With the vegetable-tanned leather, however, it is clear that the vegetable tans almost completely prevent reaction of FDNB with α-amino groups, and it is impossible to use this method for determining hydrolytic breakdown.

The N-terminal residues found in the leathers are given in Table IV. The same amino acids were found as N-terminal residues in all the samples,

TABLE IV

N-TERMINAL RESIDUES IN DETERIORATED COLLAGENS AND LEATHERS

(mmole/100 g. collagen)

	Collagen					Formaldehyde-tanned			High chrome-tanned			Mimosa-tanned		Myrobalans tanned and Stored at 60° C. for 6 Weeks
			Stored at 60° C. for 6 Weeks											
	Control (Not Stored)	Stored at 40° C. for 16 Weeks	Whole	Insoluble in 5*M* Acetic Acid	Soluble in 5*M* Acetic Acid	Control	Stored at 40° C. for 16 Weeks	Stored at 60° C. for 6 Weeks	Control	Stored at 40° C. for 16 Weeks	Stored at 60° C. for 6 Weeks	Con-trol	Stored at 60° C. for 6 Weeks	
Aspartic acid	0.016	0.016	0.027	0.093	0.094	0.004	0.007	0.009	0.006	0.024	0.048	trace	0.002	trace
Glutamic acid	0.007	0.005	0.008	0.007	0.064	0.004	0.004	0.008	0.003	0.009	0.023	"	0.001	"
Serine	0.035	0.008	0.018	0.013	0.065	0.005	0.004	0.007	0.005	0.019	0.063	"	0.002	"
Threonine	0.008	0.011	0.009	0.005	0.064	0.005	0.007	0.004	0.003	0.062	0.061	"	0.002	"
Glycine	0.082	0.088	0.054	0.044	0.38	0.030	0.026	0.040	0.012	0.090	0.34	"	0.016	"
Alanine	0.016	0.036	0.027	–	0.18	trace	0.010	0.013	trace	0.082	0.21	"	trace	"
Valine	0.012	0.033	– 0.2*	– 0.1*	0.083	trace	0.007	0.007	trace	0.036	0.035	"	trace	"
Phenylalanine	0.050	0.14	–	–	0.32	trace	0.008	0.012	trace	0.12	0.27	"	trace	"
Total	0.226	0.337	0.343	0.262	1.25	0.048	0.073	0.100	0.029	0.436	1.05		0.023	
Mean weight per mole of N-terminal residue	442,000	300,000	291,000	382,000	80,000	2,080,000	1,370,000	1,000,000	3,350,000	226,000	95,200		4,350,000	

*Unidentified artifact interfered with separation.

and the pattern of α-amino-groups released during deterioration is very similar. Glycine generally contributes the largest number of N-terminal residues, with alanine and, perhaps rather surprisingly in view of the small amount present in collagen, phenylalanine contributing very nearly as many, and in one or two cases even more.

There was very little breakdown observed in the collagen, probably because the pH of this material was above 4.5. The fraction soluble in $5M$ acetic acid contained most of the α-amino groups released, and the mean weight per mole of terminal residues in this fraction was about the same as in a good grade gelatin (6). With formaldehyde-tanned leather there was only a small increase in N-terminal residues in storage. On the other hand, with the chrome leather there were quite large increases in N-terminal residues, especially after storage at 60°C. This deteriorated leather contained almost as many N-terminal residues as the material extracted from collagen by $5M$ acetic acid, but it was only soluble to the extent of 2.1% in this solvent. Presumably this is due to the stabilizing effect of the chrome tan.

The amounts of ϵ-DNP-lysine found in the different samples showed considerable variations. Determinations of free lysine in the DNP-materials showed corresponding variations, and in most cases the sum of the free lysine and the ϵ-DNP-lysine approximated closely the total lysine of the original collagen (Table V). Since it has been shown that ϵ-DNP-lysine does not

TABLE V

REACTED AND UNREACTED LYSINE IN MATERIALS TREATED WITH FLUORODINITROBENZENE

(mmole/100 g. collagen)

(The collagen has a total lysine content of 27.1 mmole/100 g., calculated from Table VI)

	ϵ-DNP-Lysine	Free Lysine	Total
Collagen			
No storage	29.0	4.6	33.6
Stored at 40°C.	16.4	11.6	28.0
Stored at 60°C.	6.5	20.8	27.3
Formaldehyde-tanned			
No storage	10.5	11.6	22.1
Stored at 40°C.	17.1	10.1	27.2
Stored at 60°C.	9.2	10.2	19.2
Chrome-tanned-4.6% Cr_2O_3			
No storage	2.5	26.6	29 1
Stored at 40°C.	13.4	15.4	28.8
Stored at 60°C.	6.0	22.3	28.3
Mimosa-tanned			
No storage	2.5	23.0	25.5

break down to lysine on hydrolysis (14), it may be concluded that the variations in the amounts of ε-DNP-lysine are due to differences in the reactivity of the lysine towards FDNB and not to variations in the recovery. The very low ε-DNP-lysine in the chrome-tanned leather suggests the possibility of some reaction of chrome tan with free amino groups, but it may be due to steric effects. In the formaldehyde-tanned leather there is a tendency for the sum of the lysine and ε-DNP-lysine to be lower than the total lysine content of the collagen, suggesting that not all the lysine which has reacted with formaldehyde is recovered after hydrolysis (see also page 214). Storage decreases the accessibility of the α-amino groups in collagen to reaction with FDNB, but with leathers it tends to have the reverse effect.

Amino acid analysis.—The results of the amino acid analysis carried out on the original collagen, on the samples of collagen stored at 60°C. and 40°C., and on a sample of material extracted by 5*M* acetic acid from the collagen stored at 60°C. are given in Table VI as amino acid nitrogen as % of total nitrogen and in Table VII as grams of amino acid residue per 100 g. moisture-free protein.

TABLE VI

AMINO ACID ANALYSIS OF COLLAGEN SAMPLES

(Nitrogen as % of total nitrogen)

	Collagen Control	Collagen Stored 16 Weeks at 40° C.	Collagen Stored 6 Weeks at 60° C.	Acetic Acid Extract of Collagen Stored at 60° C.
Hydroxyproline	8.04	8.28	7.64	4.12
Aspartic acid	3.61	3.58	3.69	4.01
Threonine	1.26	1.29	1.35	1.51
Serine	2.66	2.61	2.70	2.68
Glutamic acid	5.72	5.98	5.61	5.73
Proline	10.51	10.93	10.71	10.03
Glycine	28.42	26.26	26.67	23.23
Alanine	9.41	8.94	8.91	6.77
Valine	1.60	1.49	1.58	1.71
Methionine	0.38	0.32	0.35	0.25
Iso-leucine	0.99	0.94	0.96	1.08
Leucine	2.03	1.94	1.97	2.11
Tyrosine	0.26	0.22	0.26	0.33
Phenylalanine	1.02	1.74	1.00	1.09
Histidine	1.17	0.61	1.28	1.38
Lysine	4.30	4.00	4.03	4.27
Hydroxylysine	0.72	0.66	0.69	0.76
Arginine	15.74	15.31	15.80	15.58
Ammonia	3.59	4.52	3.65	4.95
Total	101.43	99.62	98.85	91.95

The recovery of nitrogen as amino acids is essentially complete in the original and deteriorated collagens, and the results show that there are no marked changes in amino acid composition resulting from storage. The weight recovery as amino acid residues* (Table VII) is, however, decreased, indicating the presence of a considerable amount of material of low nitrogen content especially in the collagen stored at 60°C. If this arose from deamnation of amino acids, it could represent a considerable breakdown of the collagen molecule.

TABLE VII

AMINO ACID ANALYSIS OF COLLAGEN SAMPLES

(Grams of amino acid residue per 100 g. protein)

	Collagen Control	Collagen Stored 16 Weeks at 40° C.	Collagen Stored 6 Weeks at 60° C.	Acetic Acid Extract of Collagen Stored at 60° C.
Total N	17.8	17.6	17.1	15.9
Hydroxyproline	11.56	11.77	10.55	5.29
Aspartic acid	5.28	5.18	5.18	5.24
Threonine	1.62	1.64	1.67	1.73
Serine	2.94	2.85	2.87	2.65
Glutamic acid	9.38	9.70	8.84	8.40
Proline	12.97	13.34	12.70	11.06
Glycine	20.61	18.82	18.58	15.04
Alanine	8.50	7.98	7.73	5.46
Valine	2.01	1.86	1.91	1.91
Methionine	0.63	0.53	0.56	0.37
Iso-leucine	1.42	1.34	1.33	1.39
Leucine	2.92	2.76	2.72	2.71
Tyrosine	0.54	0.45	0.52	0.61
Phenylalanine	1.91	1.74	1.80	1.82
Histidine	0.68	0.61	0.71	0.72
Lysine	3.50	3.22	3.15	3.11
Hydroxylysine	0.66	0.60	0.61	0.62
Arginine	7.81	7.51	7.53	6.91
Total	94.94	91.90	88.96	75.04

The weight recovery of the material extracted with 5*M* acetic acid is even lower (75%), and the nitrogen recovery is correspondingly low. However, since this fraction only represents 10% of the total weight of the deteriorated collagen, by no means all the low-nitrogen-containing material is concentrated in it. The amino acid composition of this more soluble fraction differs

*The rather low weight recovery for the original collagen is usual for this type of material which has received an alkaline pretreatment. It is probably due to the association with the protein of organic acid ions, in this case acetate, derived from the neutralizing procedure.

appreciably from that of collagen. The hydroxyproline is very low, the glycine and alanine are low, and the proline is slightly low, while most of the other amino acids tend to be slightly high. It seems that it is the more polar part of the molecule which is going into solution, while the more ordered part believed to contain repeating units of glycine-proline-hydroxyproline is relatively less affected during storage.

Chrome tanning and vegetable tanning had no effect on the amino acid composition, so the results are not quoted. In all cases nitrogen recovery was essentially complete; weight recovery could not be readily assessed because of the presence of tan. In the formaldehyde-tanned leather the tyrosine was completely absent, and there was a slight reduction in lysine content, from 27 mmole/100 g., to 24.5 mmole/100 g. Single runs on the deteriorated samples did not show any appreciable differences from the original, and as the changes in collagen were small, it was not considered worth further investigation.

DISCUSSION

The deterioration of the leathers during storage followed the same general trend as in previous experiments. The vegetable-tanned leathers were more damaged than the chrome leathers, especially at the high temperature. The formaldehyde-tanned leather was the least damaged. It did not lose much strength, and the soluble nitrogen was low, even in 5*M* acetic acid. The myrobalans-tanned leather was the most damaged of the vegetable-tanned leathers, and the quebracho-tanned leather the least damaged.

All the results indicate that breakdown of the protein occurs to varying extents during the exposure of collagen and leather to moist heat. With the collagen and the vegetable-tanned leathers the soluble nitrogen became high, but as in previous experiments, there was little increase in the soluble nitrogen of the chrome leathers, even when the leather appeared quite badly damaged and the determination of N-terminal residues indicated appreciable hydrolytic breakdown of the protein. With the formaldehyde-tanned leather the soluble nitrogen and N-terminal residue determinations provided little evidence for protein breakdown.

The results in Table IV indicate that hydrolytic scission of peptide bonds plays an important part in the protein breakdown. In the collagen this breakdown is quite small, and even though its soluble nitrogen is high, the 5*M* acetic acid extract only has about the same number of N-terminal residues as a good quality gelatin (6). Hence the solubility may be due to a molecular disorganization similar to that occurring during gelatinization rather than being completely due to hydrolytic breakdown.

The chrome tan prevents most of the free amino groups from reacting with FDNB in the original leather, but it is clear that there is considerably more hydrolytic breakdown in the deteriorated leather than in the collagen. The values for ε-DNP-lysine indicate that some blockage may still occur in the deteriorated leather, and breakdown may be even more extensive than the results suggest.

Formaldehyde appears to block the original N-terminal groups, and there is apparently very little release of new groups during storage. It may be that the formaldehyde reacts with new groups as they are released, but since there is a loss of formaldehyde during storage, this seems unlikely. The recovery of lysine from formaldehyde-tanned leather is apparently incomplete, especially after storage at 60°C., suggesting that either not all the formaldehyde is removed from combination with amino groups during hydrolysis or that, if it is, some of the amino groups are lost in the process. Work now in progress using ^{14}C-labelled formaldehyde suggests the former (15).

The wide variations in the reactivity of the ε-amino groups to FDNB are difficult to understand. It was observed during the amino acid analysis of DNP-leathers that the amounts of histidine and hydroxylysine showed similar variations to those of lysine, suggesting that some common steric factor may be involved.

Unfortunately the FDNB method cannot at the moment be used for determining N-terminal residues in vegetable-tanned leathers due to interference by the tans. Attempts to extract vegetable tans completely from deteriorated leathers have so far been unsuccessful, and it has been impossible to overcome the interference.

The results of analysis for amino acid composition do not indicate much change during storage. The composition of the fraction of collagen soluble in 5*M* acetic acid suggests that the less regular regions of the molecule most readily go into solution, leaving the more ordered parts of the molecule, containing glycine, proline, and hydroxyproline, insoluble. There is, however, a considerable amount of indirect evidence that some form of breakdown other than hydrolytic has occurred. The increases in volatile nitrogen in acetic acid extracts, the slight general losses of amino acids, indicated by the low weight recovery of the deteriorated collagens, and particularly the large proportion of material of low nitrogen content in the extract from collagen stored at 60°C. all point to some type of breakdown involving deamination. Bacterial damage cannot be completely ruled out, but since the results at 60°C. are similar to those at 40°C., it seems more likely to be of a chemical nature. It is probable that this is oxidative, but further evidence is not available.

A general loss of even 1% of each amino acid could result in a reduction to a mean molecular weight of about 10,000, giving a product which would almost certainly be readily soluble. Such losses are within the experimental error of the method ($\pm$ 3% of the amino acid found), and clearly any breakdown from oxidation or deamination is more likely to be found by looking for the products of the breakdown rather than by determining the undamaged amino-acids.

The pH of water extract and shrinkage temperature show the large variability that was expected from previous results. The high pH and low shrinkage temperature of the myrobalans-tanned leather stored at 40°C. are probably a result of the breakdown to produce the high volatile nitrogen shown by this leather, but the cause of this particular breakdown is not clear. Otherwise there is no obvious connection between changes in these measurements and the other changes observed.

There is, however, an apparent correlation between the amount of hydrolytic breakdown and the original pH of the leather. It is well known that leather tends to lack resistance to acid atmospheres, but the breakdown observed in this experiment in many cases occurs at pH values at which hydrolysis of the hide protein was previously believed not to occur (16).

The amount of hydrolytic breakdown in the vegetable-tanned leathers cannot be determined by the FDNB method, but there must have been an appreciable amount to produce the high soluble nitrogen. It appears that the resistance of a leather to deterioration under the conditions used for storage in this experiment depends, first, on its pH, which controls the amount of hydrolytic breakdown, and second, on the ability of the tanning material to hold together the products of breakdown and prevent disorganization of the molecular structure.

REFERENCES

1. Bowes, J. H., and Raistrick, A. S. Part I. *JALCA*, **56**, 606 (1961).
2. Raistrick, A. S. Part II. *JALCA*, **56**, 616 (1961).
3. Bowes, J. H., and Raistrick, A. S., Part III. *JALCA*, **56**, 632 (1961).
4. ———. Part IV. *JALCA*, **58**, 190 (1963).
5. ———. Moss, J. A. *Biochem. J.*, **55**, 735 (1953).
6. Courts, A. *Biochem. J.*, **58**, 70, 74 (1954).
7. Cassel, J. M. *JALCA*, **53**, 507 (1958).
8. Bowes, J. H. *Progress in Leather Science, 1920-45*. BLMRA, 1948, p. 515.
9. Sanger, F. *Biochem. J.*, **39**, 507 (1945).
10. Porter, R. R., and Sanger, F. *Ibid.*, **42**, 287 (1948).
11. Rao, K. R., and Sober, H. A. *J. Amer. Chem. Soc.*, **76**, 1328 (1954).
12. Moore, S., Spackman, D. H., and Stein, W. H. *Anal. Chem.*, **30**, 1185 (1958).
13. Hannig, K. *Clin. Chim. Acta*, **4**, 51 (1959).
14. Moss, J. A. Ph.D. Thesis, Univ. London, 1959.
15. Ellis, M. J. Unpublished.
16. Phillips, H. *J. Roy. Soc. Arts*, **102**, 824 (1954).

Received November 27, 1963.